Self-assembling dairy proteins for the production of novel bionanomaterials

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Abstract: The casein family of proteins shows promise for the formation of value added bionanomaterials. A co-product that is rich in casein is produced in excess as a by-product of the dairy and dairy processing industries. Caseins derived from dairy dissolved air flotation (DAF)-derived casein sludge were found to possess different fundamental properties. When analysed by RP-HPLC, the hydrophobic profile of the DAF-derived caseins were found to be different from that of skim milk powder (SMP) caseins. Rheological studies found that DAF-derived caseins were more viscous than those from SMP, which supported the idea that the DAF process may have induced some additional cross-linking between neighbouring caseins. This was also justified by the high apparent molecular weight of some caseins when separated on SDS-PAGE and identified by MALDI-TOF MS. The DAF-derived caseins were found to self-assemble, and produce cohesive and flexible films when dried under controlled conditions. These observations indicate that the DAF-derived caseins may have distinct features enabling the production of novel value-added bionanomaterials.

Keywords: casein; protein; bionanomaterial; bioplastic; film; DAF; dissolved air flotation.


Biographical notes: Kate Ryder has an MSc in Biochemistry from the University of Otago. Currently, she has just completed a PhD in Bioengineering at the University of Otago. Her current research is in the development of biomaterials from a protein co-product of the dairy industry.
Caseins are the primary protein family found in bovine milk [1]. They possess the ability to self-assemble into higher order structures and are dynamically flexible. This gives them the potential for use in the production of bionanomaterials such as hydrogels, drug encapsulation systems and nanospun fibres for the cosmetic and nutrition sectors [2]. The amphipathic nature of milk proteins may be exploited in the formation of a stable film, used in the commercial production of aerated foods or in firefighting foams [3]. The self-associating abilities of caseins lead to the potential ability to form cohesive bioplastics that can be used as an alternative to petrochemical plastics for functional packaging and coating materials [4]. These materials are environmentally sustainable making them more attractive as added value biomaterials. However the mechanical properties still fall short of many petrochemical plastics [5].

Dairy factories generate considerable quantities of milk protein-containing wastewater. The high organic load of this wastewater is of environmental concern and requires treatment. One process, dissolved air flotation (DAF) may be used to generate a cleaner water effluent, but also produces a casein-rich sludge material which may be dried to a powder [6]. This DAF-casein is a complex composite that comprises proteins, sugars, lipids and minerals. All of these components may influence the rheological and mechanical properties of the materials. While previous milk-based biomaterials have focused on the use of the protein component alone, the use of DAF-casein as a composite allows for the potential to produce a biomaterial with unique properties while utilising a waste stream.
2 Methods

**SDS-PAGE analysis of proteins.** One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D-SDS-PAGE) was conducted using Bolt gradient (4-12%) Bis-Tris gels (Invitrogen, CA, USA). Samples were prepared as described in the manufacturers protocols with Bolt LDS sample buffer and Bolt sample reducing agent. Electrophoresis was conducted in 1× Bolt MES SDS running buffer at 164 V for 34 min. Gels were stained overnight in SimplyBlue SafeStain (Invitrogen) and destained in Milli-Q water prior to imaging.

**MALDI-TOF MS.** Bands of interest were excised from SDS-PAGE gels. Following destaining and drying, the selected gel pieces were rehydrated and incubated with trypsin in ammonium bicarbonate buffer (trypsin: protein ratio of 1 : 2) at 37°C overnight. Digest peptides extracted from the gel were used for tandem matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF-MS). Mass spectrometry was conducted in the Centre for Protein Research, University of Otago.

**Reversed phase high-performance liquid chromatography (RP-HPLC).** The DAF-casein powder (supplied by Callaghan Innovation, Wellington, New Zealand) and SMP were reconstituted at 20 mg.mL⁻¹ in 50 mM KH₂PO₄ buffer, pH 7.5. Samples were combined with 1 mL 0.1% (v/v) trifluoroacetic acid (TFA), 20% (v/v) and 200 μL was injected on a C4 Jupiter column (250 × 4.6 mm i.d., 300 Å, 5 μm particle size (Phenomenex, New Zealand). The elution gradient used was 25–45% solvent B, over 30 min, 45–60% B over 30 min, at a flow rate of 1.0 mL.min⁻¹ (solvent A, 0.1% (v/v) TFA, solvent B 0.1% (v/v) TFA, 90% (v/v) acetonitrile). The column effluent was monitored at 280 nm and 215 nm and collected in 1 mL fractions for further analysis, if required.

**Rheological study.** All rheological measurements of DAF-casein solutions were carried out using a Haake™ rotational rheometer fitted with a 60 mm 1° titanium cone. The temperature was controlled at 20 ± 0.5°C. The shear rate was controlled between 0 and 200 s⁻¹ over a period of 300 s and 100 data points were collected. The distance was set at 52 μm. Formulations were prepared three times and measured in triplicate on new aliquots.

**Bioplastic film formation.** Films were formed by drying DAF-casein solutions (2% w/v) in 140 mm diameter Teflon trays (Welch Fluorocarbon, NH, USA) at ambient temperature, to yield 6.36 mg.cm⁻²⁻¹ films.

3 Results and discussion

DAF-casein showed a similar protein profile to SMP on SDS-PAGE (Figure 1), however, the DAF-casein showed significant smearing on the gel (Figure 1). MALDI-TOF MS analysis of the DAF-casein sample identified the caseins and major whey proteins as predicted. Additionally, some of the proteins were identified in regions associated with molecular weights higher than the theoretical mass of the predicted proteins. For example, αs1-casein (24.4 kDa) and β-lactoglobulin (19.9 kDa) were also identified in the 80 kDa region of the gel. This may be an indication of the formation of higher order protein aggregates or crosslinked proteins, which may also be responsible for the smearing pattern observed.
Figure 1 1D-SDS-PAGE analysis of reconstituted DAF powder compared to reconstituted SMP. Lane 1: Novex Sharp Pre-stained Protein Standard (Invitrogen); Lane 2: DAF powder (20 mg.mL⁻¹, 5 μL); Lane 3: reconstituted SMP (100 mg.mL⁻¹, 2 μL). Proteins in circled bands were identified with MALDI-TOF MS. Protein identities of each band are given in the accompanying table (see online version for colours).

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
<th>Mass (Da)</th>
<th>Species</th>
<th>pI</th>
<th>% Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>αs-casein</td>
<td>24,420</td>
<td>Bos taurus</td>
<td>4.85</td>
<td>33%</td>
</tr>
<tr>
<td>2</td>
<td>β-lactoglobulin</td>
<td>19,908</td>
<td>Bos taurus</td>
<td>4.85</td>
<td>19%</td>
</tr>
<tr>
<td>3</td>
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<td>24,420</td>
<td>Bos taurus</td>
<td>4.85</td>
<td>38%</td>
</tr>
<tr>
<td>4</td>
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<td>Bos taurus</td>
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<td>23%</td>
</tr>
<tr>
<td>5</td>
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<td>Bos taurus</td>
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<td>42%</td>
</tr>
<tr>
<td>6</td>
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<td>14%</td>
</tr>
<tr>
<td>7</td>
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<td>Bos taurus</td>
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</tr>
<tr>
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<td>Bos taurus</td>
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<tr>
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<tr>
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<td>Bos taurus</td>
<td>4.80</td>
<td>36%</td>
</tr>
</tbody>
</table>

The hydrophobic behaviour of the proteins in DAF-casein was compared to SMP (Figure 2). DAF-casein proteins eluted slightly later with reduced peak resolution. DAF-casein showed only two major peaks compared to three in milk powder, suggesting alteration of the hydrophobic characteristics of some DAF proteins. This may be due to the formation of crosslinks between proteins of varying hydrophobicity. SMP was chosen as a comparison due to similarities in the drying processes, and enabled consideration of any differences observed being due to either the drying process or the DAF process.

Figure 2 RP-HPLC of DAF-casein and SMP. DAF-casein (blue), SMP (green), buffer B gradient (grey) (see online version for colours).

When comparing the viscosity of solutions at 200 s⁻¹, the DAF-casein showed a significantly higher viscosity indicating a greater resistance to deformation (2.7 ± 0.24 mPas compared to 1.2 ± 0.04 mPas) (Figure 3). The higher viscosity of the DAF-casein compared to the milk solution also supports the idea of higher order crosslinking occurring. The crosslinking would result in larger protein aggregates intertwining and interacting together, forming a more viscous solution.
Self-assembling dairy proteins for the production of novel bionanomaterials

Figure 3  Rheological comparison of DAF-casein and SMP. Shear rate ($\gamma$, s$^{-1}$) vs viscosity ($\eta$, Pas). DAF-casein (blue), SMP (green). The shaded area represents the mean ± one standard deviation ($n = 3$) (see online version for colours)

The unique fundamental and mechanical properties of DAF-casein indicate the potential for novel value-added bionanomaterial formation. DAF-casein exhibits a self-assembling capability and forms a thin and continuous bioplastic film when dry (Figure 4(A)), showing that the self-assembling properties of caseins remain following the DAF process. In comparison, SMP films break upon drying (Figure 4(B)). This could be due to the lower lipid content which may be acting as a plasticiser in the DAF-casein films. Blending DAF-casein with other proteins and polysaccharides improves the homogeneity, transparency, flexibility and dimensional stability of the films (Figure 4(C) and (D)). Current work is underway to evaluate the use of cross-linkers and plasticisers to enhance the mechanical properties.

Figure 4  Self-assembled bioplastic films formed from DAF treated casein proteins. Panel A: 100% DAF-casein film. Panel B: 100% SMP. Panel C: 60% DAF-casein, 40% gelatin composite film. Panel D: 60% DAF-casein, 40% cellulose composite film (see online version for colours)

4 Conclusions

Milk solids can be efficiently harvested from dairy wastewater by a DAF process to generate a casein rich co-product (DAF-casein). The DAF-casein is available in high volume and has potential utilisation in the production of value-added biomaterials. The co-product has a similar protein composition compared to that of commercial milk
samples. However, physicochemical properties as shown by SDS-PAGE, RP-HPLC and rheology indicate that the properties of the DAF caseins are different from those of SMP. Solutions of DAF-casein showed an increased viscosity and a change in hydrophobicity compared to milk powder. The DAF-casein was found to retain self-assembling properties and form films, indicating the potential for the production of bioplastic films. Future work is directed to the production and evaluation of the mechanical properties of these films.

Acknowledgements

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References